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## Aquaporin modulators: a patent review (2010-2015)

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Aquaporin modulators: a patent review (2010-2015)

Abstract

**Introduction:** Since the discovery of aquaporin-1 (AQP1) as water channel more than 2000 articles, reviews and chapters have been published. The wide tissue expression, functional and biological roles have documented the major and essential physiological importance of these channels both in health and disease. Thus, over the past years, studies have revealed essential importance of aquaporins in mammalian pathophysiology revealing aquaporins as potential drug targets.

**Areas covered:** Starting from a brief description of the main structural and functional features of aquaporins, their roles in physiology and pathophysiology of different human diseases, this review describes the main classes of small molecules and biologicals patented, published from 2010 to 2015, able to regulate AQPs for diagnostic and therapeutic applications.

**Expert opinion:** Several patents report on AQPs modulators, mostly inhibitors, and related pharmaceutical formulations, to be used for treatments of water imbalance disorders, such as edema. Noteworthy, a unique class of gold-based compounds as selective inhibitors of aquaglyceroporin isoforms may provide new chemical tools for therapeutic applications, especially in cancer. AQP4-targeted therapies for neuromyelitis optica, enhancement of AQP2 function for nephrogenic diabetes insipidus and AQP1-5 gene transfer for the Sjogren’s syndrome represent promising therapies that deserve further investigation by clinical trials.

**Keywords:** aquaporins, small-molecule inhibitors, gold compounds, biologicals, cancer, edema, nephrogenic diabetes insipidus, neuromyelitis optica.

**Article highlights:**

- Aquaporins (AQPs) are membrane channels involved in a wide range of physiological functions and human diseases. Modulators with confirmed selectivity, their pharmaceutical compositions and methods for use in diagnostic and treatment represent promising strategies for treatment of AQP-related pathologies.
- Compounds and methods for detecting and treating renal diseases and immune-inflammatory diseases such as neuromyelitis optica (NMO) are the topic of numerous patent applications. Development of monoclonal antibodies or blockers of IgG-AQP4 will possibly be successful for prevention and treatment of NMO lesions.
- A number of patents describe small molecule AQPs modulators and pharmaceutical formulations for treatments of water imbalance disorders and edema. However, the selectivity of the modulators should be guaranteed to avoid side effects.
- Gold-based compounds showed selective inhibition of aquaglyceroporin isoforms and may be further optimized for pharmaceutical applications particularly for cancer treatment.
- Upregulation of AQP1, 2 and 5 may prove beneficial for treatment of loss-of-function aquaporin diseases such as renal disease, nephrogenic diabetes insipidus (NDI), salivary and lacrimal gland dysfunction, as well as obesity among others.

1 – Introduction

Water is the major component of cells and tissues throughout all forms of life. Fluxes of water and solutes through cell membranes and epithelia are essential for osmoregulation and energy homeostasis. The existence of water channels embedded in the lipid bilayer is crucial for facilitating water flow across cell membranes assuring the rapid equilibration of osmotic gradients necessary in many situations.

25 years ago the fundamental discovery and characterization of an abundant protein of the erythrocyte membrane termed Aquaporin-1 (AQP1) represented a paradigm shift in the understanding of molecular, membrane and organism water transport. Worldwide spread studies have shown that AQP1 is a member of an ancient family of water and solute-permeable membrane proteins – the AQUAPORINS - which have since then be demonstrated to be ubiquitous in all domains of life <sup>[1]</sup>. Nowadays, it is well established that AQPs are membrane channels that facilitate the permeation of water and small solutes across membranes, driven by osmotic or solute gradients <sup>[2]</sup>. In mammals, the 13 aquaporin isoforms identified so far (AQP0-12) are expressed in a wide range of tissues and are involved in many biological functions. Based on their primary sequences and permeation specificities, AQPs are classified as i) orthodox aquaporins, considered selective waterstrict water channels (AQP0, 1, 2, 4, 5, 6, 8), although (AQP6 and AQP8 beingare also involved in the transport of anions <sup>[3]</sup>, NH<sub>3</sub>ammonia <sup>[4]</sup> and H<sub>2</sub>O<sub>2</sub> <sup>[5]</sup>), ii) aquaglyceroporins, permeable to water and small uncharged solutes like glycerol (AQP3, 7, 9, 10), and iii) unorthodox aquaporins, found mostly intracellularly, with lower sequence homology and permeability still unclear (AQP11, 12) <sup>[6]</sup>.

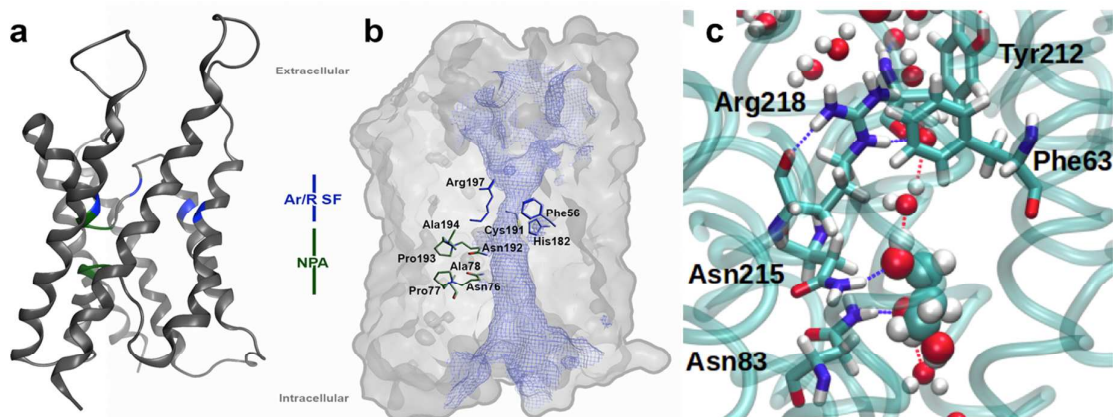
AQPs are composed by around 320 amino acid residues with approximately 28 kDa, architected in membranes as tetramers. Each monomer is formed by six transmembrane domains and behaves as an independent pore <sup>[7]</sup>.

The most remarkable feature of AQP channels is their high selectivity and efficiency on water or glycerol permeation, excluding ions and protons <sup>[8]</sup>. Apart from water and glycerol, a

number of other permeants such as urea, ammonia, hydrogen peroxide, carbon dioxide, metalloids, nitric oxide <sup>[9]</sup> and even ions <sup>[10]</sup> were reported to permeate specific AQPs, although the mechanism of permeation is still obscure.

AQPs share a common protein fold, with the typical six membrane-spanning helices surrounding the 20-Å-long and 3-4-Å-wide amphipathic channel, plus two half-helices with their positive, N-terminal ends located at the centre of the protein and their C-terminal ends pointing towards the intracellular side of the membrane. A feature of AQPs in all organisms is the presence of two constriction sites (Fig. 1): i) an aromatic/arginine selectivity filter (ar/R SF) near the periplasmic/extracellular entrance, that determines the size of molecules allowed to pass through and provides distinguishing features that identify the subfamilies; and ii) a second constriction site composed by two conserved asparagine-proline-alanine (NPA) sequence motifs (variants exist, for example NPC in the case of AQP11 and NPT in AQP12, respectively), located at the N-terminal ends of the two half-helices, at the centre of the channel. In orthodox aquaporins the ar/R SF is very narrow, constituted usually by four aminoacid residues, typically arginine, phenylalanine, histidine and a fourth residue (e.g a cysteine). In aquaglyceroporins the ar/R SF is broader due to the existence of only three amino acid residues, normally arginine, phenylalanine and tryptophan or threonine. The SF (Fig. 1) provides distinguishing features that identify the subfamilies <sup>[11]</sup>: thus, differences in the actual size available for passage of solutes, but also the type of residues (e.g. histidines or cysteines in the ar/SF, instead of aromatic rings) allow to distinguish between isoforms and their affinity towards different solutes.





**Figure 1** – Structure of the AQP1 water channel (a , b); monomeric form of the channel, displayed in ribbon (a) and surface (b), with the channel surface represented as a blue mesh, and SF and NPA regions indicated; (c) passage of water and glycerol molecules through AQP3.

Due to their numerous roles in physiology, AQPs are essential membrane channels involved in crucial metabolic processes and expressed in almost all tissues <sup>[12]</sup>. The functional importance of AQPs in mammalian pathophysiology has been extensively studied by analysing the phenotype of transgenic knockout mice lacking these water channels. According to the reported studies, AQPs participate in many physiological and pathophysiological processes that include renal water absorption, brain water homeostasis, tumour angiogenesis, fat metabolism, liver gluconeogenesis and reproduction <sup>[13, 14]</sup>.

As an example, in the renal collecting duct, water reabsorption is regulated by the antidiuretic hormone vasopressin (arginine vasopressin, AVP). Binding of this hormone to the vasopressin V2 receptor (V2R) leads to insertion of AQP2 water channels in the apical membrane, thereby allowing water reabsorption from the pro-urine to the interstitium and consequent urine concentration <sup>[15-17]</sup>. Defects preventing the insertion of AQP2 into the plasma membrane cause diabetes insipidus. The disease can be acquired or inherited, and is characterized by polyuria and polydipsia. Inversely, up-regulation of the system causing a predominant localization of AQP2 in the plasma membrane leads to excessive water retention and hyponatremia as in the syndrome of inappropriate antidiuretic hormone secretion (SIADH), late stage heart failure or liver cirrhosis <sup>[18]</sup>. The disorder nephrogenic diabetes insipidus (NDI) is characterized by the kidney's inability to concentrate pro-urine in response to AVP, which is mostly acquired due to electrolyte disturbances

or lithium therapy. Alternatively, NDI is inherited in an X-linked or autosomal fashion due to mutations in the genes encoding V2R or AQP2, respectively<sup>[19]</sup>.

The mammalian 'aquaglyceroporins' regulate glycerol content in epidermal, fat and other tissues, and appear to be involved in skin hydration, cell proliferation, carcinogenesis and fat metabolism. The functional significance of glycerol transport by aquaglyceroporins has been the subject of several studies. For example, these channels represent pathways for glycerol absorption by the gastrointestinal tract, for partial glycerol reabsorption or secretion by the kidney and for entry/exit processes in adipocytes, depending on the metabolic state of the organism<sup>[20, 21]</sup>. The glycerol thus made available can be used also as fuel by the heart and skeletal muscle and as a substrate for gluconeogenesis by the liver. Aquaglyceroporins in the capillaries may function as channels that modulate glycerol flow into and out of the bloodstream.

Moreover, glycerol permeation in adipocyte membranes carried out by aquaglyceroporins seems to be intrinsically related to the mechanisms of obesity and type 2 diabetes<sup>[20, 22, 23]</sup>. Impaired glycerol transport through plasma membrane AQP7 has been correlated with triglyceride accumulation and obesity onset. AQP7 is the main gateway facilitating glycerol release from adipocytes although other glycerol channels such as AQP3, 9, 10 and the most recently described AQP11, also contribute to glycerol efflux from fat depots<sup>[23, 24]</sup>. Circulating plasma glycerol is then introduced in hepatocytes via AQP9 that represents the primary route for glycerol uptake in hepatocytes<sup>[25]</sup>. In the liver, glycerol constitutes a direct source of glycerol-3-phosphate for triglyceride synthesis and an important substrate for hepatic gluconeogenesis during fasting<sup>[26]</sup>. Thus, the coordinated regulation of aquaglyceroporins in adipocytes and hepatocytes plays a key role in maintaining the control of fat accumulation in adipose tissue and liver, as well as whole-body glucose homeostasis<sup>[23]</sup>.

Due to their roles in these glycerol pathways, AQPs may represent potential therapeutic targets in the management of obesity and associated metabolic disorders. Interestingly, thiazolidinediones, insulin sensitizer drugs for the treatment of type-2 diabetes and agonists of peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ), have revealed an upregulating effect on AQP7 in murine 3T3-L1 cells and in OLETF rats<sup>[27]</sup>. Recently, the peptide Apelin-13 was



demonstrated to reduce the lipid accumulation in hypertrophic adipocytes *in vitro* by increasing AQP7 expression, which indicates it could be used as an AQP modulator in the treatment of obesity<sup>[28]</sup>. A novel small molecule inhibitor of AQP9, HTS13286, has been identified and recognized to be effective in reducing glycerol gluconeogenesis in primary hepatocyte cultures<sup>[29]</sup>. However, at present, its therapeutic use cannot be recommended due to the high doses required and the low solubility in 1% DMSO aqueous solution.

~~For example~~ Among the other aquaglyceroporins, AQP3 was also shown of great importance in different pathways. For example, AQP3-facilitated glycerol transport in skin has been shown to be an important determinant of epidermal and stratum corneum hydration.<sup>[30]</sup> Thus, mice lacking AQP3, which is normally expressed in the basal layer of proliferating keratinocytes in epidermis, manifest reduced stratum corneum hydration and skin elasticity, and impaired stratum corneum biosynthesis and wound healing<sup>[31]</sup>. Recognizing the relationship between AQP3 expression and skin moisturization, several companies have marketed cosmetics (such as Eucerin®, Be+®, HydrAction®) containing ingredients claimed to increase AQP3 expression.

Broad range of evidence also suggests that these protein isoforms have a role in cell proliferation by various different mechanisms, including: i) allowing fast cell volume regulation during cell division; ii) affecting progression of cell cycle; iii) helping maintaining the balance between proliferation and apoptosis, and iv) cross-talking with other cell membrane proteins or transcription factors that, in turn, modulate progression of the cell cycle or regulate biosynthesis pathways of cell structural components<sup>[32]</sup>. However, so far no clear mechanism disclosing the interplay between AQPs and cell proliferation has emerged, and new experiments designed specifically to address these challenging research questions are necessary.

Notably, other studies have shown the relationship between ~~aquaglyceroporins~~ ~~aquaporins~~ AQPs expression and cancer development<sup>[33]</sup>. Recent publications reported that a few AQPs might enhance cell proliferation, migration and survival in a variety of cancer malignancies<sup>[33, 34]</sup>, suggesting AQPs as promising drug targets and its modulators as useful anti-tumor agents<sup>[35]</sup>. Thus, for example AQP1 and AQP5 are highly expressed in several different tumor cell types<sup>[33, 34, 36]</sup> where they are believed to be involved in rapid changes in cell shape and volume that

require rapid flow of water into and out of the cell. In addition, enhanced expression of aquaglyceroporin isoforms have been reported, among others, in colorectal carcinogenesis [37], human lung [38], gastric adenocarcinomas [39] and human skin squamous cell carcinomas [40], as well as with metastasis [41]. In this case, it was hypothesized that different mechanisms, other than volume changes, are in place to explain AQP's participation in the cell proliferation process, most likely related to the necessity of glycerol for key metabolic reactions in cells [32, 36].

It is worth mentioning that AQP's are also found in disease-causing bacteria (AqpZ and GlpF in *Escherichia coli*), in protozoan parasites that cause malaria (PfAQP in *P. falciparum*) and toxoplasmosis (TgAQP1 in *Toxoplasma gondii*), as well as in parasitic worms that cause Chagas disease (TcAQP1, TcAQP2 and TcAQP3 in *Trypanosoma Cruzi*), sleeping sickness (TbAQP1, TbAQP2 and TbAQP3 in *Trypanosoma brucei*) and schistosomiasis (SmAQP in *Schistosoma mansoni*) [42]. In addition to facilitating water transport, many parasitic AQP's are permeable to glycerol and lactate. Thus, both host and parasite AQP's are potential drug targets for treatment of parasitic infections.

A totally different pathophysiological condition where an aquaporin isoform is involved is Neuromyelitis Optica (NMO). NMO is a relapsing autoimmune disorder of the central nervous system usually associated with autoantibodies against AQP4 water channel, expressed predominantly on astrocytes. NMO pathogenesis involves NMO-IgG binding to AQP4, which causes complement-dependent cytotoxicity and antibody-dependent cell-mediated cytotoxicity [43]. Although NMO used to be considered a variant of multiple sclerosis (MS), it is now recognized as a distinct clinical entity with a unique pathophysiology and differing epidemiology, genetics, treatment, and outcomes from MS [43, 44]. The consequences of astrocyte damage include inflammation with cytokine release and infiltration of granulocytes and macrophages, disruption of the blood-brain barrier, and injury to oligodendrocytes and neurons. Current NMO treatments include general immunosuppressive agents, B-cell depletion, and plasma exchange. New therapeutics are needed with improved efficacy and reduced long-term side effects.

Overall, it is evident that due to their important biological roles and involvement in several pathophysiological conditions, there is a great translational potential in aquaporin-based

diagnostics and therapeutics <sup>[35]</sup>. Within this frame, this review illustrates patent applications published in the past 5 years (summarized in Table 1) claiming either small compounds or biologicals as aquaporin modulators for therapeutic applications and/or methods for diagnosis and treatment of aquaporin related disorders. Data were researched using the Espacenet database (<http://worldwide.espacenet.com>).

**2 – Small molecules as aquaporin modulators**

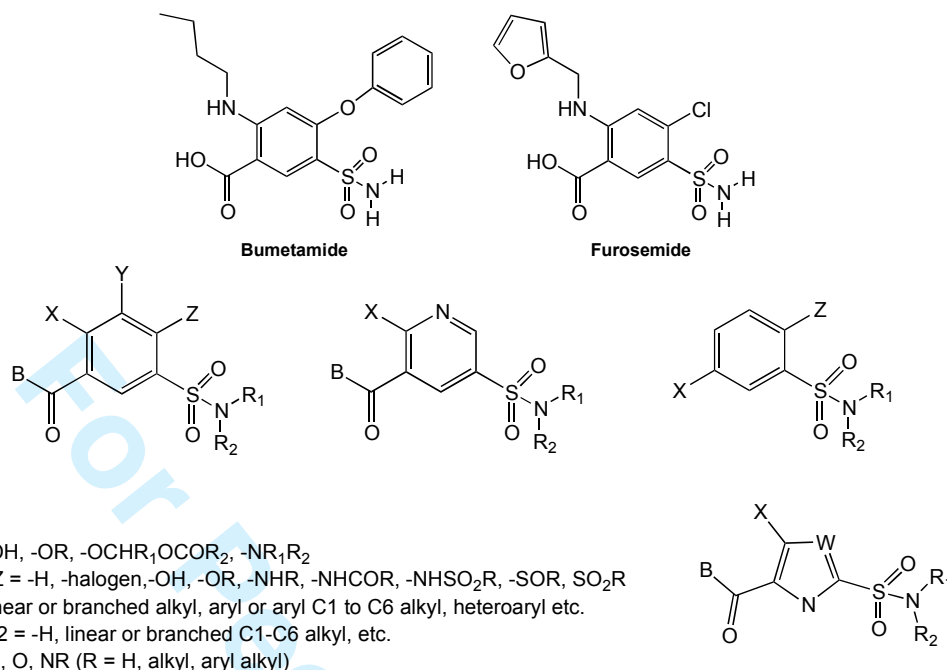
Concerning small molecules as AQPs inhibitors with therapeutic applications, a few classes have been so far reported and patented in the last years. It is worth mentioning that here we will focus only on those molecules patented between 2010-2016, however several other small molecule AQPs modulators have been published during the years. Since the latter are outside the scope of this expert opinion, we refer the reader to other reviews, including from Beitz et al. <sup>[45]</sup> and Verkman et al. <sup>[35]</sup>.

2.1. - Modulators of orthodox AQPs

The first family of patented compounds, ~~reported~~ in 2008, include sulfonamides commercially used as loop diuretics, such as bumetanide, furosemide, and other substituted 3-carboxy-aryl-sulfonamide derivatives or analogues, as well as heteroaryl sulphonamides <sup>[46]</sup> (Fig. 2). The same compounds were further described in a US patent in 2014 <sup>[46]</sup>. Specifically, the inventors have discovered that such compounds are blockers, and in some cases stimulators, of aquaporin function in a *Xenopus* oocyte expression system. Thus, compounds having the basic core structures reported in Figure 2 have been demonstrated to selectively modulate AQP1 and AQP4 function in cells. Seeing the roles of these isoforms in physiology, their inhibitors may lead to new ways to significantly decrease the formation of edema after ischemic stroke and acute brain injury. Furthermore, this class of aquaporin modulators may allow treatment of other diseases associated with or mediated by aquaporins, such as glaucoma, macular degeneration, pulmonary disorders, and certain types of cancers, such as brain tumors.

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3 In the same patent, new ways to introduce bumetanide and furosemide into target cells  
4 where these compounds have significant activity on aquaporins, such as AQP4 are disclosed,  
5 including design of prodrug forms of these compounds that produce molecules that are  
6 intracellularly active on aquaporins when taken up by cells. For example, the acetoxymethyl pro-  
7 drug of furosemide may be employed as an AQP4 modulator and antagonist. The above  
8 compound is one example of a compound that has a new use for modulating aquaporin activity  
9 when bio-converted to a known drug (furosemide) inside of a cell. Similar prodrugs based on either  
10 bumetanide and furosemide or other known loop diuretics, which undergo activation inside of a  
11 target cell, are contemplated and may be synthesized as described in the patent.  
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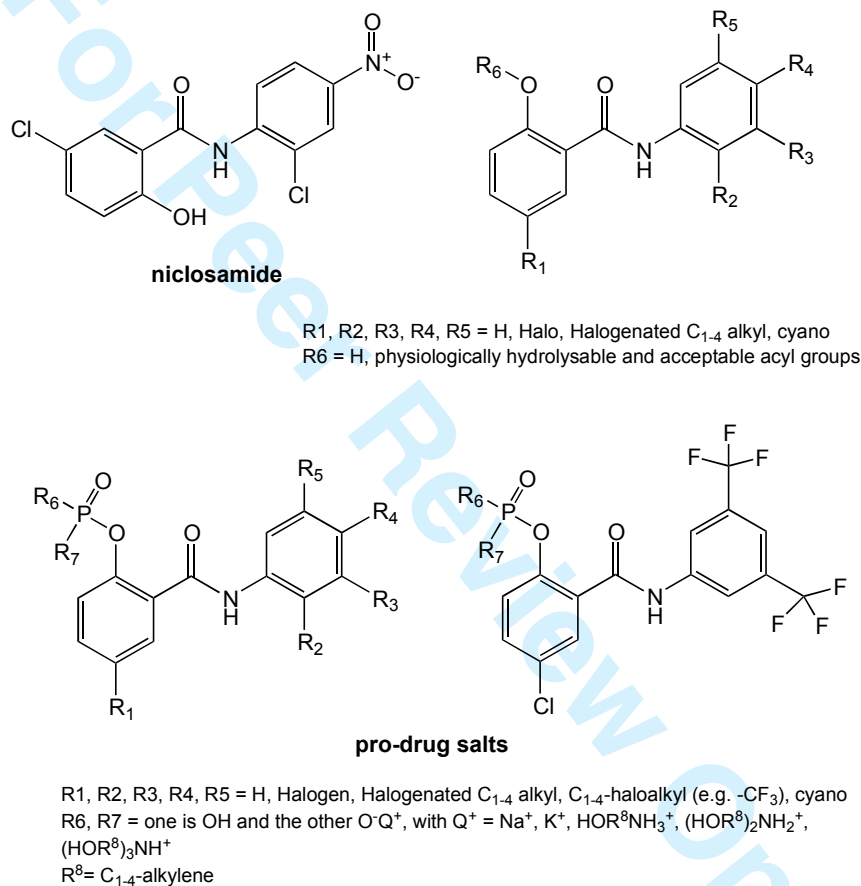
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21 It should be mentioned that, recently, a paper reporting on the AQP1 inhibition efficacy of  
22 12 previously reported small-molecule AQP1 inhibitors, including some of those in the above  
23 mentioned patent, showed no activity of the compounds on water permeation in human and rat  
24 erythrocytes natively expressing AQP1<sup>[47]</sup>. The authors conclude that the sulfhydryl-reactive,  
25 heavy metal-containing compounds are the only confirmed small-molecule AQP1 inhibitors  
26 reported to date and highlight the need of using robust, sensitive assays for screening and  
27 computational work to identify useful inhibitors.  
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**Figure 2** – Structure of sulfonamide-based compounds as AQP1 and AQP4 modulators.

In 2013 Pelletier et al. patented inhibitors of AQP2 and AQP4 including phenylbenzamide-type of compounds such as niclosamide (5-chloro-*N*-(2-chloro-4-nitrophenyl)-2-hydroxybenzamide), an antihelminthic drug to treat tapeworm, and compounds of general formula reported in Figure 3, for the prophylaxis, treatment and control of AQP-mediated diseases involving water imbalance <sup>[48]</sup>. Specifically, the compounds were proposed for the treatment of various types of edema mediated by AQP4, such as the one due to trauma or ischemic stroke (edema of the brain and spinal cord), the edema associated with glioma, meningitis, epileptic seizures, infections, metabolic disorders, hypoxia, hepatic intoxication, as well as edema caused by microgravity and/or radiation exposure, and due to invasive neurosurgical procedures among others. Authors proposed the same inhibitors (specifically for AQP2) for the treatment of hyponatremia and excess fluid retention by inhibiting water uptake in the kidneys. In addition, a subsequent patent claims the use of the above-mentioned compounds for the treatment of glioblastoma <sup>[49]</sup>. Pharmaceutical formulations and novel pro-drug salts (Fig. 3) of the same compounds were also patented for different therapeutic applications as described above <sup>[50]</sup>.

The invention further claim the above mentioned compounds to be used as positive controls for AQP2 and AQP4 inhibition in high-throughput assays, comprising measuring the response of an AQP-expressing cell population versus a control cell population to a hypertonic or hypotonic solution in the presence or absence of a test compound, via fluorescence microscopy (calcein-based assay).



**Figure 3** – Structure of AQP2 and AQP4 phenylbenzamide-type inhibitors and related pro-drug salts.

## 2.2. - Aquaglyceroporin inhibitors

Among the small molecule AQPs inhibitors, the family of coordination gold-based compounds is unique in being selective for inhibiting aquaglyceroporin isoforms. In fact, in 2013 Soveral and Casini patented a number of gold(III) complexes with N-donor ligands as AQP3, AQP7 and AQP9 inhibitors <sup>[51]</sup>, the lead compound being the water-soluble gold(III) coordination

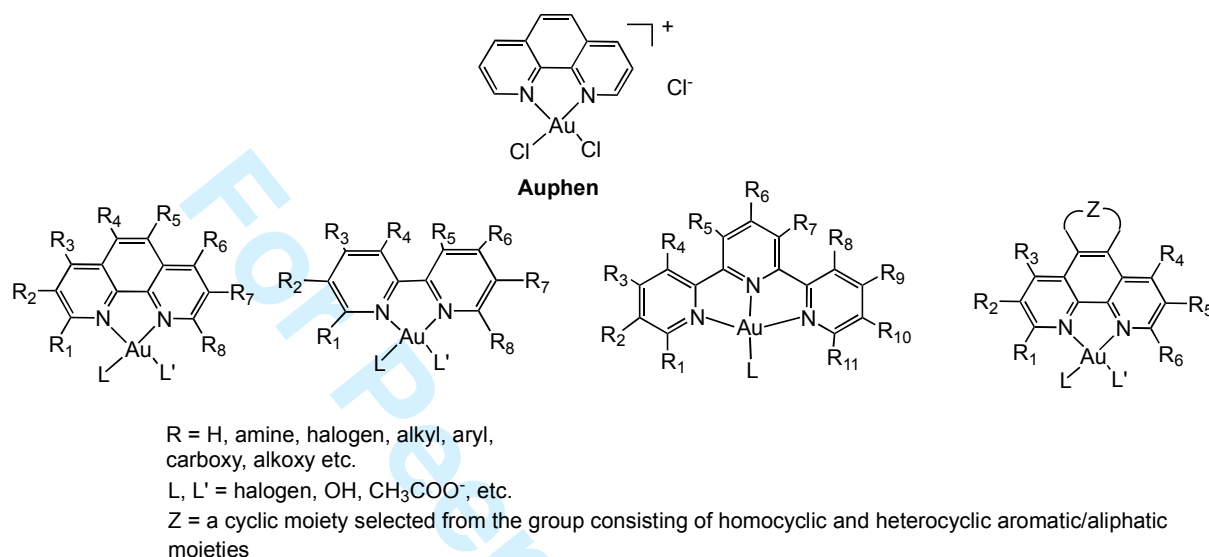


compound,  $[\text{Au}(\text{phen})\text{Cl}_2]\text{Cl}$  (phen = 1,10-phenantroline, Auphen) (Fig. 4). Notably, the compounds did not have any effect on the permeability of the orthodox water channel AQP1 in different cellular models. The overall series of gold complexes included compounds of the type reported in Figure 4, where the main scaffolds are substituted-1,10-phenantroline, substituted-2,2'-bipyridine, substituted-2,2',2''-terpyridine, as well as polypyridyl ligands. Each of  $\text{R}_1$  through  $\text{R}_{11}$  (Fig. 4) is independently selected from the group consisting of H; aliphatic, heteroaliphatic, aromatic, heteroaromatic, aliphatic-aromatic, heteroaliphatic-heteroaromatic, cycloaliphatic, and heterocycloaliphatic groups comprising up to four C-atoms; amines (e.g.  $\text{NH}_2$ , aliphatic amines  $-\text{R}-\text{NH}_2$ ); halogens (e.g. chloride, iodide); moieties with hydroxyl functional groups (e.g.  $-\text{OH}$  or  $-\text{Y}-\text{OH}$ ); ether containing moieties of general formula  $-\text{Y}-\text{O}-\text{Y}'$ ; carbonyl containing moieties ( $-\text{Y}-\text{C}(\text{O})\text{OH}$ ); or of amide bonds ( $-\text{Y}-\text{C}(\text{O})\text{N}-\text{Y}'$ ); sulfonamidic groups; nitrile/nitro groups, and peptide moieties, wherein Y and Y' are independently selected from aliphatic, heteroaliphatic, aromatic, heteroaromatic, aliphatic-aromatic, heteroaliphatic-heteroaromatic, cycloaliphatic, and heterocycloaliphatic groups comprising up to four C-atoms. Moreover, L and L' are independently selected from the group consisting of halogen, hydroxyl, acetate, phosphane, and thiol-bearing groups (e.g. thio-sugars, cysteine and methionine groups); and Z is a cyclic moiety selected from the group consisting of homocyclic and heterocyclic aromatic/aliphatic moieties, preferably 6,6-, 5,6- or 6,5-fused bi-homocyclic and bi-heterocyclic aromatic/aliphatic moieties, wherein the heterocyclic moieties may include nitrogen, oxygen and/or sulfur atoms.

A preferred embodiment of the invention provided metal-based inhibitory modulators of cellular transmembrane aquaglyceroporins comprising organogold compounds, where the organic ligands are for example 6-(1,1-dimethylbenzyl)-2,2'-bipyridine), substituted-2-phenyl-pyridine or 2-[[[(dimethylamino)methyl]phenyl] moiety and 1,3-bis(pyridin-2-ylmethyl)benzene is preferable, together with phosphine groups and thiolate ligands.

The invention claims such gold-based complexes in the treatment, prophylaxis and prevention of a number of clinical conditions, such as wound healing, tumours and cancer growth, angiogenesis, pathological skin conditions, obesity, kidney disorders, salivary gland disorders,

allergic diseases, glaucoma, brain edema and epilepsy. Overall, these inhibitors are claimed to manufacture pharmaceutical compounds, cosmetics and diagnostic kits.

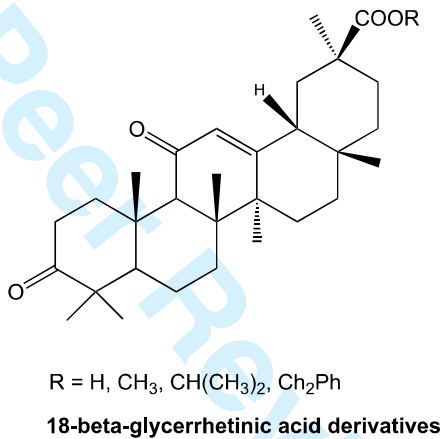


**Figure 4** – Gold(III) complexes as selective aquaglyceroporin inhibitors.

Interestingly, among the aquaglyceroporins, the modulation (inhibition or induction) of AQP9 expression or activity by different substances was claimed to be of therapeutic interest in the treatment of bone diseases associated with unbalanced osteoclast differentiation<sup>[52]</sup>. According to this invention, inhibition of osteoclast differentiation by AQP9 modulators may have a therapeutically beneficial effect in pathological conditions associated with osteoclast formation and differentiation such as excessive bone loss. Along differentiation of precursor mononuclear cells to form multinucleated osteoclasts, the volume of mature osteoclasts increases dramatically and the additional cytosolic volume is favored by the enhanced water influx promoted by aquaporins. The inventors found that an increase in AQP9 expression precedes osteoclast precursors fusion into osteoclasts, and thus by modulating AQP9 expression or activity, uncontrolled effects of osteoclast formation could be prevented. For example, the invention encompassed the use of an AQP9 inhibitor, such as phloretin (3-(4-hydroxyphenyl)-1-(2,4,6-trihydroxyphenyl)propiophenone) among others, for prevention or treatment of conditions associated with excessive bone resorption (osteoporosis, rheumatoid arthritis, bone cancer, bone infections, periodontal disease, Paget's

disease) or an AQP9 inducer for prevention or treatment of conditions associated with osteoclast deficiency, which may result in overly dense bones (osteopetrosis).

Concerning AQPs activators, in 2013, a derivative of 18-β-Glycyrrhetic acid (Fig. 5) and its pharmaceutically acceptable formulations was patented, which was shown to be able to induce AQP3 overexpression in fibroblasts and human keratinocytes <sup>[53]</sup> . Thus, the compound is proposed as treatment to increase the cell number in the above mentioned cell types, as well as for wound healing, and more in general for treatment of diseases caused by aquaporin deficiency.



**Figure 5** – AQP3 modulators (agents causing protein overexpression).

**3 – Biologicals for aquaporin detection and regulation**

Several patents have been published claiming new methods for diagnosis and treatment of aquaporin related diseases. In particular, kidney malfunction including nephrogenic diabetes insipidus (NDI) and the autoimmune disease neuromyelitis optica (NMO), are established aquaporinopathies that prompted the development of AQP-based therapeutics.

[3.1 - Modulation of tissue water homeostasis](#)

[Treatment of renal disease and loss of renal function through aquaporin modulation has been the goal of patented compounds and methods.](#) The major functions of the kidney include the elimination of metabolic waste products and the regulation of water, electrolyte and acid-base

balance. Filtration of blood in the glomeruli produces 180 L/day of filtrate that is subsequently reabsorbed in the tubular system. Most of the water, salt and dissolved substances are reabsorbed to the capillary system and only around 1% of the volume (approximately 1.5 L/day) is excreted as urine. The massive water reabsorption is facilitated by aquaporins differentially expressed along the tubular epithelial membranes. In particular, AQP1 expressed at the proximal tubule apical membranes and AQP2 expressed at the kidney collecting duct and being regulated by vasopressin, play a key role in urine concentration and body-water homeostasis <sup>[54]</sup>.

The renal collecting duct water permeability is controlled by the hormone vasopressin through regulation of AQP2, either by modulating the trafficking of AQP2 at the apical plasma membrane (short-term regulation) or by increasing total cellular abundance of AQP2 protein (long-term regulation). Following the binding of vasopressin to its V2R at the plasma membrane, the rise in cAMP activates protein kinase A (PKA) that in turn phosphorylates AQP2 and triggers its insertion at the membrane. Loss-of-function mutations of both V2R and AQP2 are the cause of nephrogenic diabetes insipidus (NDI, the inability of the kidney to respond to vasopressin stimulation and consequent impairment of urine concentration). Inversely, gain-of-function mutations of the VR2 are associated with the nephrogenic syndrome of inappropriate diuresis (NSIAD) characterized by water retention and hyponatremia.

The most common form of NDI is acquired NDI. One of the main causes is long-term lithium treatment of bipolar disorders. This causes acquired NDI in up to 40% of the patients <sup>[18]</sup>. Lithium inhibits adenylyl cyclases and thus cAMP synthesis and, consequently, the cAMP-dependent signaling that controls AQP2 trafficking and expression in the collecting duct, resulting in a pronounced vasopressin-resistant polyuria and inability to concentrate urine. The diuretics thiazide combined with amiloride can have profound antidiuretic effects in patients with lithium-induced NDI, an effect that has been associated with increased AQP2 expression. Captopril (an angiotensin-converting enzyme inhibitor) and spironolactone (a mineralocorticoid receptor blocker) also induce a decreased urine production in rats with lithium-induced NDI, probably by a mechanism involving AQP2 upregulation <sup>[55]</sup>.

Desmopressin (DDAVP), a synthetic vasopressin analog, is currently used to treat diabetes

insipidus, bedwetting or nocturia and blood clotting disorders by a mechanism that involves binding to the V2R. However due to its side effects, its use has been limited. Vaptans, a new class of drugs developed for the treatment of hyponatremia<sup>[56]</sup>, are non-peptide vasopressin receptor antagonists causing water diuresis (aquaresis) thus increasing blood sodium. These selective V2R antagonists were proved to generate aquaresis, an electrolyte-sparing excretion of free water, which results in normalization of serum sodium concentration<sup>[57]</sup>. Some vaptans were tested in animal models and in human trials and a few have been approved by Food and Drug Administration (FDA) in the United States for the treatment of hyponatremia; however several side effects including thirst, headache, hypokalemia, polyuria, vomiting and diarrhea have been reported. More studies are needed to better define vaptans long-term safety and efficacy.

Long-term treatment with vasopressin increases the total abundance of cellular AQP2<sup>[15]</sup>, which is the resulted balance of protein synthesis and protein removal via degradation or exosomal secretion. AQP2 in urinary extracellular vesicles (exosomes) appears to correlate with the plasma vasopressin concentration<sup>[58]</sup> and AQP2 is being measured as a biomarker for diagnosis and treatment of water-balance disorders, for example, congestive heart failure and liver cirrhosis. However, controversial results and considerations in the literature raise the possibility that urinary AQP2 excretion is more a reflection of its actual abundance in the apical cell membrane of kidney collecting duct principal cells rather than being related with vasopressin<sup>[59, 60]</sup>. In the latter scenario, urinary AQP2 can be considered a reliable biomarker only of AQP2-dependent renal disorders. More investigation on the molecular mechanisms and pathways responsible of AQP delivery into the urine shall be paramount.

In 2013, a patent describing a method for differentiating embryonic stem cells into cells expressing AQP1 that can transport water in a manner similar to renal epithelial cells was published<sup>[61]</sup>. The method involves culturing the embryonic stem cells in renal-specific growth medium and in the presence of an extracellular matrix molecule to provide nephrogenic differentiation conditions that induce differentiation into cells expressing AQP1. Thus, this method attempts to provide a cell source for the therapy of lost renal function (renal failure, nephrosis,

Bright's disease and glomerulitis) through bioartificial devices, cell therapies to replace epithelial tissues and tissue engineering with application in regenerative medicine.

Treatment of ~~nephrogenic diabetes insipidus (NDI, a genetic disorder caused by loss of function AQP2 mutations)~~ NDI and edema (the swelling of tissues due to excessive fluid accumulation) through the modulation of aquaporin in tissues by administration of relaxin, is the claim of another recent patent <sup>[62]</sup>. Systemic edema is commonly associated with heart, liver and kidney diseases, and is caused by increased salt and water retention in the body that induce fluid accumulation in interstitial spaces, where it appears as edema. Cerebral edema is believed to involve AQP4, the most abundant aquaporin within the brain that appears to play a role in brain water physiology, brain edema and hydrocephalus <sup>[63, 64]</sup>. Several AQPs are implicated in other forms of edema (ocular edema, pulmonary edema, peripheral edema and systemic edema), and thus modulating aquaporin channel expression in mammals has been proposed to revert fluid accumulation associated with edema <sup>[65]</sup>. In this invention, methods of modulating aquaporin channels through the administration of relaxin, a polypeptide hormone similar to insulin that is secreted from the ovary in high concentrations during pregnancy, are described. Relaxin classic roles are related with tissue remodelling processes that occur in the extracellular matrix of the cervix and vagina before the start of labour in animals. However, the inventors have shown that relaxin is capable of modulating aquaporins (up- and/or down-regulating and/or modifying gene and/or protein expression) in different tissues, thereby changing the permeability of biological membranes to water and consequently changing the fluid content of body tissues and organs. The patent anticipates that relaxin is a key regulatory factor in the production of concentrated urine in the kidney, and that it acts directly on the inner medulla collecting duct cells to stimulate intracellular cAMP and mediate AQP2 trafficking to the apical membrane, thus promoting water reabsorption. In addition, it is contemplated that relaxin ameliorates systemic edema through a mechanism that regulates and/or modulates the water channels. After administering relaxin intravenously or subcutaneously a measurable reduction of fluid accumulation within 24 hours or less after the onset of treatment is predicted to occur.



Several novel potential targets and mechanisms that could be targeted for treatment of dysregulated tissue water homeostasis have been reported. A recent review summarizing the currently available pharmacotherapies and new options for the treatment of water balance disorders that are not encompassed by the herein patent review can be found in <sup>[18]</sup>.

3.2. - Diagnosis and treatment of autoimmune diseases

Compositions and methods for the treatment of neuromyelitis optica, an autoimmune disease caused by AQP4-specific antibodies, has been by far the topic of the majority of the patents published in the last five years. Neuromyelitis optica (NMO), a rare disease also known as Devic's disease, is an autoimmune, inflammatory disorder in which the inflammatory demyelination of the central nervous system causing attacks of optic neuritis (ON) and transverse myelitis (TM), often recurrent, cause relapse and associated disability. ~~the immune system attacks the optical nerves and spinal cord, producing an inflammation of the optic nerve (optic neuritis) and the spinal cord (myelitis).~~ The main symptoms are loss of vision and spinal cord function that can lead to muscle weakness, reduced sensation or loss of bladder and bowel control, paraplegia and blindness. Some patients have limited or atypical forms of the disease usually termed as NMO spectrum disorders (NMOSD) <sup>[66]</sup>. Similarly to multiple sclerosis, there is immune-mediated destruction of the myelin surrounding nerve cells. However in NMO the attacks are primarily mediated by antibodies, called NMO-IgG, that target AQP4 expressed in the astrocytes cell membranes <sup>[43]</sup>.

AQP4 is expressed in astrocytes in two major forms: a long (M1) isoform with translation initiation in Met-1, and a shorter (M23) isoform with translation initiation at Met-23. M23 AQP4 assembles in membranes as regular square arrays called orthogonal arrays of particles (OAPs) originally seen by freeze-fracture electron microscopy <sup>[67]</sup>. OAP formation by M23 results from tetramer-tetramer interactions involving residues just downstream of Met-23 at its cytoplasmic N-terminus while residues in M1 AQP4 just upstream of Met-23 disrupt this interaction. While M1 does not form OAPs on its own, it can co-assemble with M23 in hetero-tetramers that limit OAP size. The biological significance of OAP formation by AQP4 remains unknown, with speculated functions

including cell-cell adhesion, enhanced AQP4 water permeability and AQP4 polarization to astrocyte end-feet. AQP4 was found expressed in astrocytes surrounding the blood-brain barrier, a system responsible for preventing substances in the blood from crossing into the brain. The blood-brain barrier is weakened in NMO, but the mechanisms underlying NMO-IgG induced demyelination are still unknown. Treatments to alleviate the NMO symptoms are based on immunosuppression by administration of corticosteroids, azathioprine, methotrexate, cyclophosphamide, mycophenolate or mitoxantrone), depletion of CD20+ B cells (using rituximab) and plasmapheresis <sup>[68]</sup>. [Approved therapies with potential for repurposing in NMO include eculizumab \(complement inhibitor\), tocilizumab \(IL-6 receptor inhibitor\), sivelestat and cetirizine \(granulocyte inhibitors\), intravenous immunoglobulin, CD19-depleting agents, and anti-TNF therapy.](#) Clinical trials for these treatments include very small numbers of patients, and most of them are uncontrolled. [New therapeutics with improved efficacy and reduced long-term side effects are essential. Recent reviews covering the available therapeutic options in NMO can be found in](#)

[44, 69, 70]

New NMO therapeutics targeting AQP4, NMO-IgG and mediators of inflammation are being investigated. The development of monoclonal antibodies binding to AQP4 may result in different applications, such as their use as competitive binders to AQP4 displacing NMO-IgG or in the production of diagnostic kits for diagnosing NMO. However, since AQP4 antibodies cause disease, for therapeutic purposes they must be modified to render them ineffective at complement activation and immune cell recruitment. One such monoclonal antibody, aquaporumab <sup>[71]</sup>, is said to bind tightly to AQP4 without cytotoxic effector functions. In a human astrocyte derived cell line expressing AQP4, aquaporumab selectively blocked NMO-IgG binding to AQP4 and prevented NMO-IgG induced cell killing and lesion formation. In addition, aquaporumab prevented the formation of NMO lesions in *ex vivo* spinal cord slices and in mice *in vivo* without causing pathology, supporting its potential as NMO drug candidate.

Treatment of NMO with pharmaceutical compositions containing an antigen-based peptide is described in a very recent patent that relates the identification of a unique AQP4-peptide localized in loop C that is able to trigger pathogenic T cell proliferation in AQP4-knockout mice <sup>[72]</sup>.

The inventors found that administration of loop C or loop C-sequence containing peptides to wild-type mice induce an immune response triggering AQP4-reactive T cells and symptomatic optic neuritis and myelitis. However these T cells did not cause disease in AQP4-knockout mice as they lack the target. This discovery is the basis for antigen-based peptide drug design for the treatment and / or amelioration of NMO. By administering or immunizing NMO-patients with loop C or loop C-sequence containing peptides, the inventors propose a new approach where loop C peptides and AQP4-reactive T cells are new therapeutic targets as well as diagnostics tests for NMO.

A method for diagnostic of NMO based on the use of a new amino acid sequence resulting from linkage of AQP4 polypeptide fragments with strong immunogenicity, for detection of specific T cells in the blood of NMO patients was proposed <sup>[73]</sup>. In this invention the new polypeptide fragment specific to AQP4-reactive T cells is obtained through topological conformational analysis of AQP4, followed by structural analyses of the related polypeptides after combination and rearrangement. Validation of the polypeptide for NMO diagnosis is performed by the enzyme-linked immunospot technique (ELISpot) experiment, utilizing the obtained polypeptide fragment and stimulating the effector T cell in the NMO disease to secrete IL-4. The inventors claim that due to its selectivity and sensitivity, this method can be developed into a diagnosis kit for NMO diagnosis.

Another approach for NMO detection and diagnosis is the use of ligands that specifically bind to NMO-IgG <sup>[74]</sup>. Combinatorial libraries were generated providing a vast number of peptoid ligands specifically binding molecules associated with autoimmune diseases such as NMO-IgG. Screening of 100 000 peptoids using a bead-based screening approach yielded several peptoid ligands for the antigen-binding site of anti-AQP4 antibodies, that can be further explored for detection assays. In another patent, peptides with homology to human AQP4 useful in diagnosing or treating NMO, peptide composition and methods were disclosed <sup>[75]</sup>. The peptides disclosed consist on a peptide comprising a contiguous stretch of amino acids having the consensus amino acid sequence (Leu-Pro-X1-X2-Met-X3-X4-Ile-X5-X6, where X1, X2, X3, X4, X5 and X6 are any amino acids and the peptide is up to 50 amino acids in length), that bind to AQP4-specific T cells and inhibits AQP4-mediated T cell proliferation, thus being useful for NMO treatment.

Methods for diagnosis of NMO using artificial AQP4-peptides to detect autoantibodies in a

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2  
3 patient sample, for NMO diagnosis were patented <sup>[76]</sup>. In addition, diagnostic and prognostic of  
4  
5 autoimmune diseases ~~including NMO~~ can be performed using biomarkers for detecting in the  
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7 patient sample autoantibodies of AQP1, 2, 5, 7, 8 through binding to the respective protein or  
8  
9 protein fragment or cell or tissues where it is expressed <sup>[77]</sup>.

10  
11 In another approach to treat autoimmune diseases, an adeno-associated viral vector that  
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13 encodes AQP1 is administered to treat patients with the Sjogren's syndrome, an autoimmune  
14  
15 lacrimal and salivary gland disease characterized by lymphocyte infiltrates of the exocrine glands  
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17 and the production of autoantibodies <sup>[78]</sup>. Because immune cells attack and destroy the exocrine  
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19 glands that produce saliva and tears, these patients suffer from xerostomia (dry mouth) and  
20  
21 xerophthalmia (dry eyes). The decreased AQP1 expression in myoepithelial cells from gland  
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23 biopsies of Sjogren's syndrome patients led to the hypothesis that AQP1 plays a role in the  
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25 disease pathogenesis <sup>[79]</sup>. Treatment of a patient with rituximab (anti-CD20) revealed a clear  
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27 improvement of xerostomia together with a marked increase in AQP1 expression in myoepithelial  
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29 cells. In addition to AQP1, the isoform AQP5 was found abnormally expressed in acinar cells from  
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31 salivary or lacrimal glands from patients, suggesting that both AQP1 and AQP5 participate in the  
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33 pathogenesis of Sjogren's syndrome. Since the proposed mechanism for Sjogren's syndrome is  
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35 the production of autoantibodies that bind muscarinic receptors on the surface of acinar cells,  
36  
37 thereby blocking signals that trigger acinar cell function, changes of AQP1 and AQP5 expression  
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39 and localization may be linked to the inflammatory response or even be a secondary effect caused  
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41 by prolonged gland hyposalivation. A previous invention relates the use of virus-mediated transfer  
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43 of a gene encoding AQP1 to restore saliva secretion in the parotid glands of miniature pigs that  
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45 had been irradiated to destroy parotid gland function, a common side effect of radiation therapy for  
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47 head and neck cancer. Subsequently, the same strategy consisting on administering to the salivary  
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49 or lacrimal gland an adeno-associated viral vector encoding an aquaporin (AQP1 or AQP5) to  
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51 restore saliva and tear production of patients with Sjogren's syndrome has been patented <sup>[80]</sup>. If  
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53 successfully administered to the target cell, the efficient transfer of AQP1 and AQP5 genes may  
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55 restore fluid secretion and alleviate the symptoms of the disease.  
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3.3.- Diagnosis and treatment of cancer malignancies

The potential clinical utility of urine markers, aquaporin-1 (AQP1) and perilipin-2 (PLIN2, also known as adipophilin, adipose-differentiated related protein or ADFP), as biomarkers of renal cell carcinoma has been recently reported <sup>[81]</sup>. A method for detecting, diagnosing or monitoring renal cancer by detection of aquaporins in the patient biological fluids was described <sup>[82]</sup>. The method uses urine or blood as the patient sample, is thus non-invasive and allows high-throughput and point-of-care testing assays to screen large-scale populations or individuals at risk. The method is based on the detection of increased levels of AQP1 and ADFP in patients with renal cancer that were shown to diminish significantly after tumor removal, demonstrating the renal tumor origin of these proteins <sup>[83]</sup>. Detection of the proteins can be done using probes that specifically bind the protein or its fragments, such as antibodies or an antigen binding domain or their fragments, or an aptamer or an avimer. It was found that the urine of patients with kidney cancer contains increased levels of such proteins compared to normal subjects, which therefore can be used as biomarkers for diagnosing and monitoring the disease. In addition, the inventors disclose that urinary exosomes are a rich and concentrated source for polypeptides that can also be used as biomarkers for normal and tumor kidney cells.

Detecting the presence or expression of AQP4 to distinguish benign from malignant thyroid nodules was the claim of a recent patent <sup>[84]</sup>. The invention is based on the discovery that immunostaining suspected follicular neoplasm for the presence of AQP4 and H1.5 can facilitate the distinction of benign from malignant oncocytic lesions and thus is useful in deciding which thyroid nodules should be dissected or removed in thyroid surgery. Indicators of benign nodules according to the invention (positive AQP4; negative H1.5) may result in non-surgical follow-up or more limited surgery, while indicators pointing to malignancy (negative AQP4; positive H1.5) may result in more extensive surgery such as total thyroidectomy.

3.4. - Aquaporin modulation for biotechnological applications

In addition to human therapeutics, aquaporin-related inventions were also patented for animal health <sup>[85, 86]</sup>. As an example, two patents were published for the vaccination of animals and

companion animals to elicit a protective immune response against tick infestations and tick-borne pathogens transmission. These patents encompass the administration of an aquaporin isolated from cattle tick, or a composition of the tick aquaporin protein or a nucleic acid construct encoding this aquaporin, in order to elicit an immunoprotective response in livestock to the cattle tick and protection to cattle tick infestations.

Although not claiming therapeutic purposes, a recent invention involving the increase in aquaporin expression of liver cells for cryopreservation and further use in toxicological studies<sup>[87]</sup> justifies being cited in this review. Development of new drugs for human and animal use involves screening large number of compounds for safety and effectiveness, which traditionally is carried out *in vivo*. Liver cells are considered powerful tools for drug screening *in vitro*, in particular if obtained from a donor and expanded. For use in toxicity studies, the harvesting, freezing, storage/shipping and thawing of the cells is often required. The invention claims the use of a choleretic agent (such as glucagon or dibutyryl cAMP) that increases the expression of aquaporins, in particular AQP8, on cell membranes thus improving water transport properties of those cells. After contact with a cryoprotector, cells are then frozen and stored for subsequent use in toxicity screening. Enhancing the cryopreservation efficiency of liver hepatocytes while maintaining hepatocyte function would certainly contribute to screening large number of drug candidates in toxicity screening assays.

#### 4 – Expert opinion

AQPs are involved in a wide range of physiological functions and human diseases. Research data accumulated over the last years clearly indicate that AQPs can be important therapeutic targets and that their modulation can be used for treatment of several pathologies<sup>[35, 88]</sup>.

Involvements of AQP4 in NMO and of ~~mutated~~ AQP2 in NDI are the two most prominent examples of aquaporinopathies for which several patents have been published.

[Treatment of NMO has improved significantly in the recent years, due to a better](#)



understanding of the disease pathogenesis. Treatment of acute relapses by antibody removal by plasma exchange and prevention of recurrent attacks with steroid sparing immunosuppressants is current in clinical practice. Yet immunosuppression may take several months to exert maximum efficacy and many patients additionally require long-term low dose corticosteroids to maintain relapse remission. New therapies with improved efficacy and lacking the potential long-term side effects of general immunosuppressive drugs are imperative. In this sense, Development of monoclonal antibodies or small-molecule blockers of the binding of pathogenic NMO-IgG to AQP4, the alleged inducing episode in NMO pathogenesis, or utilizing bacterial enzymes that selectively inactivate IgG-class antibodies neutralizing their pathogenicity, will possibly be a reveal successful treatment for preventing development of NMO lesions in the coming years. The efficacy of aquaporin blocking antibody suggests the possibility of targeted therapy for acute and prophylactic treatment. So far no blocking therapy or engineered AQP4 antibody showed to disrupt AQP4 water channel function<sup>[69, 89]</sup> rendering unexpected any undesirable toxicity caused by impairment of AQP4 normal function. However potential side effects due to the binding of AQP4-IgG to its target itself cannot be ruled out. Recently, the C loop targeted AQP4-T cell therapy emerged as a novel strategy to target NMO. When adoptively transferred to wild-type mice, AQP4-reactive T cells cause optic neuritis and transverse myelitis, independent of antibodies, indicating its potential application in NMO therapeutics if similar effects are found in humans. Further human clinical studies are needed to provide definitive evidence of efficacy. A variety of other strategies for NMO therapy encompassing modulators of astrocyte AQP4 expression and supramolecular assembly, repurposing of approved granulocyte (neutrophil and eosinophil) inhibitors, cytokine modulators as well as optimization of drug penetration into NMO lesions, are in the pipeline. The clinical efficacy of these emerging therapies will require human trials.

Enhancement of AQP function by upregulating AQP expression or possibly by gene transfer, may prove beneficial for treatment of loss-of-function aquaporin diseases such as renal disease and NDI, salivary and lacrimal gland dysfunction and obesity among others. The availability of vaptans, new and potent orally active vasopressin receptor antagonists, has attracted

attention as a possible therapy for water balance disorders characterized by water retention and some of them are under clinical trials. However, they are ineffective in the vasopressin independent form of SIADH (caused by an activating mutation of the V2 receptor) and their effect on the vascular endothelium is unknown. Due to the wide distribution of vasopressin receptors in human tissue, a variety of adverse side effects cannot be excluded and may limit their clinical use.

A number of patents report on the properties of small molecule AQPs modulators, mostly inhibitors, and related pharmaceutical formulations, to be used for the possible treatments of water imbalance disorders and different forms of edema. However, the existence of 13 human AQP isoforms with broad tissue distributions, presents a conscious risk of possible side effects for AQP-based therapeutics unless the selectivity of the modulators is guaranteed, which is not the case for most of the patented compounds. In fact, several of the organic molecules reported so far, have not been tested for their selectivity towards most of the human isoforms. Noteworthy, a unique class of gold-based compounds as selective inhibitors of aquaglyceroporin isoforms has been described<sup>[90, 91]</sup> which may provide new chemical entities for therapeutic applications, especially in cancer disease, as well as in the treatment of obesity and insulin resistance related diseases.

Nevertheless, even the most promising scaffolds need to be further optimized for pharmaceutical applications, and detailed information of their inhibition mechanisms is essential. In this context, computational methods are essential tools to obtain the structure-activity relationship, and several homology models have recently been proposed for different aquaglyceroporin isoforms<sup>[90, 92]</sup>, also exploited in molecular dynamics (MD) studies<sup>[93]</sup>. The use of such models is recommended to achieve a deeper understanding of the key structural features involved in water/ glycerol transport, and helpful to improve AQP-targeted drug design.

With respect to inhibitors design, we are convinced that a better understanding of human aquaporins regulation in biological environments by different stimuli (e.g. gating by pH) and the identification of physiological mechanisms of water/glycerol flux modulation may open the way to new strategies to selectively target different AQPs and to achieve optimization of inhibitors<sup>[88]</sup>. For example, recently, four key amino acid residues for pH gating of human AQP3 have been identified at the interface of each monomer<sup>[94]</sup>. Protonation of such residues induces protein conformational

changes (i.e. movement of intra- and extra-cellular loops), which eventually determine channel closure. Such conformational changes may be triggered also by binding of the four residues to small molecules, such as the above mentioned gold compounds. Thus, new inhibitors design for these protein channels should also be done in the context of the overall functional aquaporin quaternary-structure, and not only oriented to achieve steric blockage of the AQPs monomer. As a matter of fact, recent MD studies applied to disclose the mechanisms of  $Hg^{2+}$  inhibition of human AQP3, support the idea that metal binding to a Cys residue induces major conformational changes in the protein structure leading to pore closure, and it is not the result of direct steric channel occlusion by the metal ion <sup>[95]</sup>.

In conclusion, there remain broad opportunities for the development of aquaporin-based diagnostics and therapeutics. While there is great promise in the development of small-molecule aquaporin-selective modulators for diagnostics and treatments, human clinical trials will ultimately establish the suitability of AQP-based therapeutics in human disease.

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**Declaration of interest**

The authors declare no conflict of interest.

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\* of interest;      \*\* of considerable interest

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**Figure Legends**

**Figure 1** – Structure of the AQP1 water channel (a , b); monomeric form of the channel, displayed in ribbon (a) and surface (b), with the channel surface represented as a blue mesh, and SF and NPA regions indicated; (c) passage of water and glycerol molecules through AQP3.

**Figure 2** – Structure of sulfonamide-based compounds as AQP1 and AQP4 modulators.

**Figure 3** – Structure of AQP2 and AQP4 phenylbenzamide-type inhibitors and related pro-drug salts.

**Figure 4** – Gold(III) complexes as selective aquaglyceroporin inhibitors.

**Figure 5** – AQP3 modulators (agents causing protein overexpression).

**Table Legend**

**Table 1** – Published patent applications and granted patents related to the development of AQPs modulators for therapeutic purposes. Patents are organized into two categories: small molecule modulators and biologicals for aquaporin detection and regulation of AQPs-related disorders, and listed in chronological order.

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Patent number	Applicants	Year	Subject
<b><i>Small molecule modulators</i></b>			
US2013137766	Fu-Jen Catholic University, TW	2013	A compound (18- $\beta$ -Glycyrrhetic acid derivative) used to prevent diseases caused by aquaporin deficiency (promoter of AQP3 expression) with application in cosmetics for skin hydration and for treatment of skin diseases (dermatitis, eczema, pruritus, etc) <sup>[53]</sup> .
WO2013169939	Aeromics, LLC, US	2013	Phenylbenzamide-type of compounds, such as niclosamide (5-chloro- <i>N</i> -(2-chloro-4-nitrophenyl)-2-hydroxybenzamide) and derivatives, as inhibitors of AQP2 and AQP4 to treat diseases related to water imbalance, including various types of edema <sup>[48]</sup> .
WO2013005170	University of Lisbon, Portugal	2013	Metal-based inhibitors of aquaglyceroporins AQP3, AQP7 and AQP9 that are useful for treating wound healing defects, tumour and cancer growth, angiogenesis, pathological skin conditions, obesity, kidney disorders, salivary gland disorders, allergic diseases, glaucoma, brain oedema and epilepsy <sup>[51]</sup> .
US8835491/ WO2008052190	University of Arizona, US	2014/ 2008	Modulators of AQP1 and AQP4; Compounds, such as bumetanide, furosemide, and other 3-carboxy-aryl sulfonamide derivatives, and methods of using them to treat related disorders, including: edema, attenuation of cerebral pressure, and maintenance of fluid balance in aquaporin-expressing tissues or organs, especially those expressing AQP4, useful for treating conditions such as stroke, brain trauma, glaucoma, macular degeneration, brain tumors, and

			cerebral infectious diseases <sup>[46]</sup> .
US8835390	Osteobuild Lda, Jerusalem	2014	Use of AQP9 modulators for the preparation of pharmaceutical formulations for treating or preventing pathological conditions associated with unbalanced osteoclast differentiation (AQP9 inhibitor e.g. phloretin) <sup>[52]</sup> .
WO2015069948	Aeromics LLC, US	2015	Phenylbenzamide-type of compounds, such as niclosamide (5-chloro- <i>N</i> -(2-chloro-4-nitrophenyl)-2-hydroxybenzamide) and derivatives, as inhibitors of AQP2 and AQP4 to treat diseases related to water imbalance, including various types of edema, as well as glioblastoma <sup>[49]</sup> .
WO2015069956	Aeromics LLC, US	2015	Pro-drug salts and pharmaceutical formulations of phenylbenzamide-type of compounds, such as niclosamide (5-chloro- <i>N</i> -(2-chloro-4-nitrophenyl)-2-hydroxybenzamide) and derivatives, as inhibitors of AQP2 and AQP4 to treat diseases related to water imbalance, including various types of edema, as well as glioblastoma <sup>[50]</sup> .
<b>Biologicals for aquaporin detection and regulation</b>			
WO2010135334	Washington University, US	2010	Detecting high levels of AQP1 in urine with antibodies probes for the diagnosis of kidney cancer <sup>[82]</sup>
WO2011112791	Corthera, Inc, US	2011	Modulation of aquaporin (AQP2, 3, 4, 5) expression in a mammal tissue by administering relaxin, for treatment of edema and nephrogenic diabetes insipidus (NDI) <sup>[62]</sup> .
WO2012145746 <a href="#">US20140170140</a>	University of <a href="#">California and University of Colorado</a> , US	2012 <a href="#">2014</a>	Compositions and methods to treat neuromyelitis optica, using antibodies binding to AQP4 either as monotherapies or in combination with immunosuppressive agents or plasmapheresis <sup>[71, 96]</sup> .
WO2012110024	Eurolmunn Medizinische Labordiagnostika, AG, DE	2012	Diagnostic kit and method for examining a human patient sample for the presence of antibodies specific to neuromyelitis optica; use of AQP4 as a starting substrate for the reaction to detect

			antibodies anti-AQP4 in patients with neuromyelitis optica <sup>[97]</sup> .
US8481316	Agency for Science, Technology and Research, Singapore	2013	A method to differentiate stem cells into renal epithelial cells expressing AQP1 that can be used to treat renal diseases (renal failure, nephrosis, Bright's disease and glomerulitis) <sup>[61]</sup> .
WO2013057599	Tzartos, GR	2013	Method to detect autoantibodies of AQP1, 2, 5, 7, 8 as biomarkers for the diagnostic and prognostic of disease <sup>[77]</sup> .
WO2013116537	Mount Sinai School of Medicine, US	2013	A method to distinguish benign from malignant thyroid nodules by detecting the presence or expression of AQP4 in oncogenic cell tissue <sup>[84]</sup> .
WO2013177142	US Government	2013	Vaccination of animals by administration of an aquaporin isolated from cattle tick to elicit an immunoprotective response in livestock against tick infestations and tick-borne pathogens transmission <sup>[85]</sup> .
WO2014154907	Protagen AG, DE	2014	Method for diagnosis of neuromyelitis optica using artificial AQP4-peptides to detect autoantibodies in a patient sample <sup>[76]</sup> .
WO2014127111	The Scripps research Institute, US	2014	Peptoids ligands that specifically bind to antibodies specific to AQP4 that cause the autoimmune disease neuromyelitis optica <sup>[74]</sup> .
US2014199333	University of California, US	2014	Human AQP4 peptides or homologs and their use for diagnosis and treatment of neuromyelitis optica <sup>[75]</sup> .
WO2014159052	US Government	2014	Composition of the tick aquaporin protein or a nucleic acid construct encoding this aquaporin for vaccination of companion animals by eliciting immune protection in livestock to the cattle tick and protection to cattle tick infestations <sup>[86]</sup> .

WO2014036468	US Government	2014	A gene transfer based method consisting on administering an adeno-associated viral vector encoding AQP1 to protect a subject from Sjogren's syndrome <sup>[80]</sup> .
US2015203553		2015	
US9091687, WO2014067229	Jun Xu, Nanjing, CN	2015	A method for diagnostic of NMO based on the use of a new amino acid sequence resulting from linkage of AQP4 polypeptide fragments with strong immunogenicity for detection of specific T cells in the blood of the patients <sup>[73]</sup> .
WO2015179360	The Johns Hopkins University, US	2015	Use of extracellular loop C peptide of AQP4 or a fragment variant of this segment in a pharmaceutical composition for screening, diagnosis and treatment of neuromyelitis optica <sup>[72]</sup> .
US2015024406		2015	Method involving the increase in aquaporin expression on cellular membrane of liver cells to improve cryopreservation efficiency and further use in toxicological studies <sup>[87]</sup> .



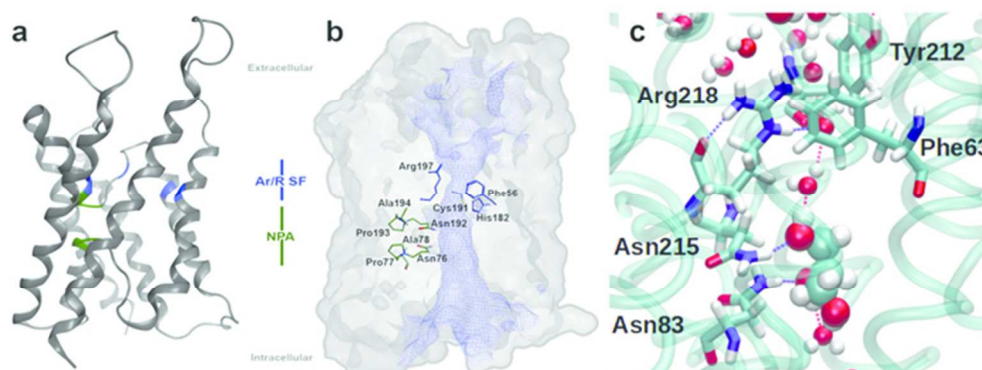


Figure 1 – Structure of the AQP1 water channel (a , b); monomeric form of the channel, displayed in ribbon (a) and surface (b), with the channel surface represented as a blue mesh, and SF and NPA regions indicated; (c) passage of water and glycerol molecules through AQP3.

Figure 1

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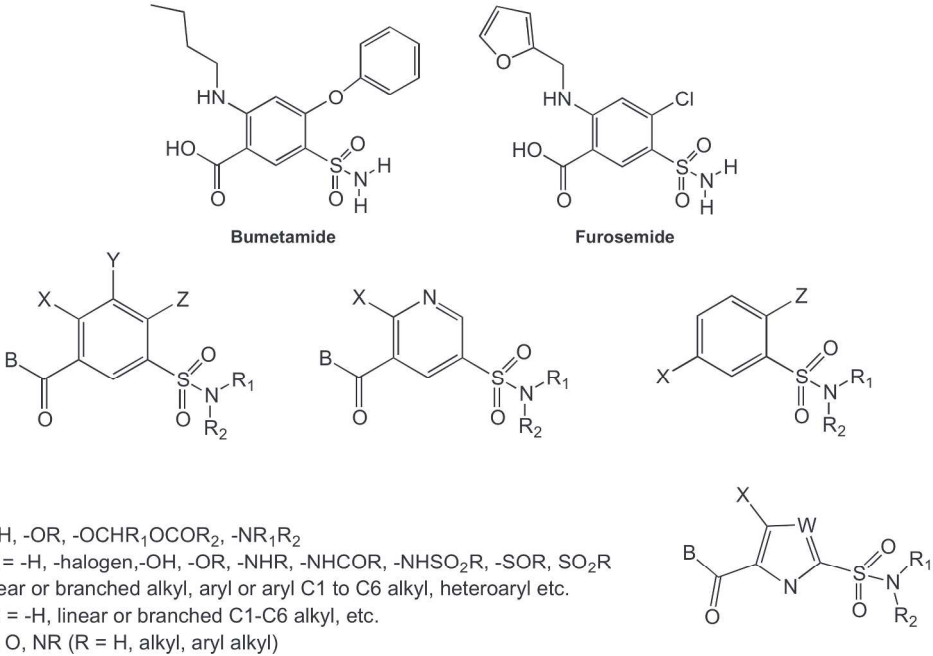


Figure 2 – Structure of sulfonamide-based compounds as AQP1 and AQP4 modulators.  
Figure 2  
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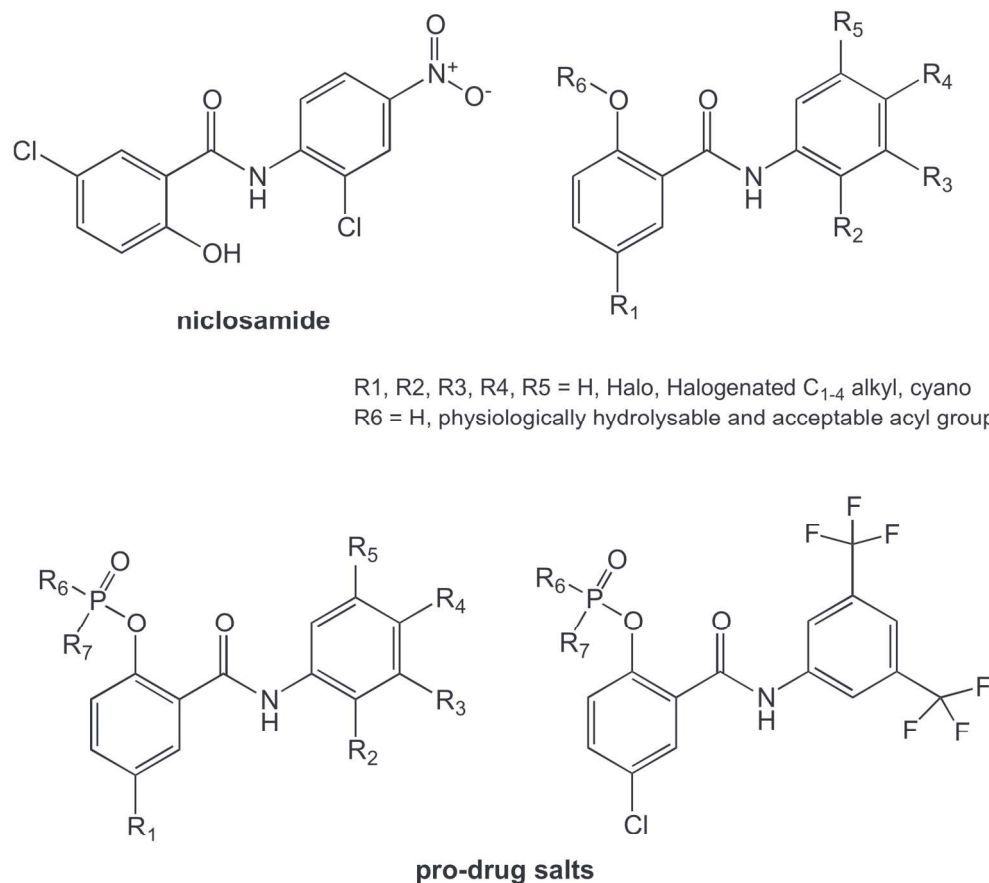


Figure 3 – Structure of AQP2 and AQP4 phenylbenzamide-type inhibitors and related pro-drug salts.

Figure 3

190x196mm (300 x 300 DPI)

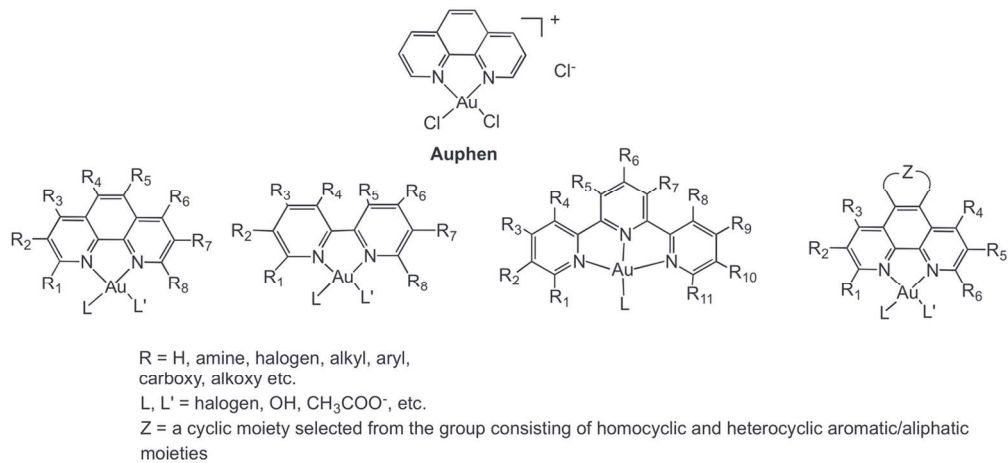
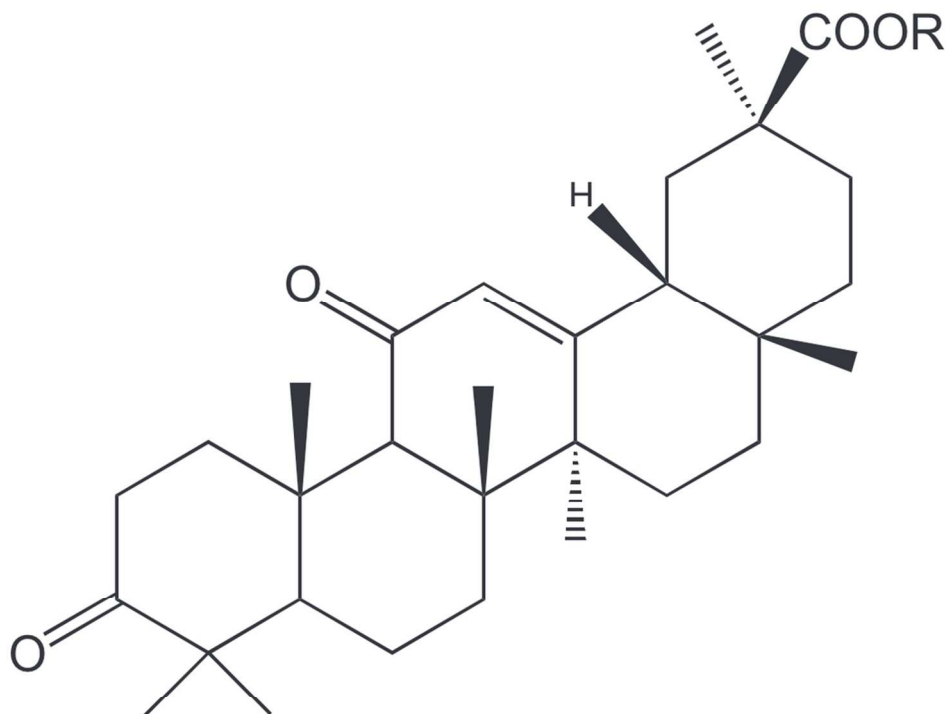


Figure 4 – Gold(III) complexes as selective aquaglyceroporin inhibitors.  
Figure 4  
124x57mm (300 x 300 DPI)



R = H, CH<sub>3</sub>, CH(CH<sub>3</sub>)<sub>2</sub>, CH<sub>2</sub>Ph

## 18-beta-glycerrhetinic acid derivatives

Figure 5 – AQP3 modulators (agents causing protein overexpression).

Figure 5

98x97mm (300 x 300 DPI)